Cellulase from the Crop of *Aplysia vaccaria* Winkler, 1955

BY

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(4 Text figures)

INTRODUCTION

The opisthobranch mollusk *Aplysia vaccaria* Winkler, 1955, is a strict herbivore; thus it would be benefited if only a small percentage of the cellulose passing through its digestive tract could be utilized.

Few animals have been shown to be capable of digesting cellulose, and it appears to be very useful to plants as a structural polysaccharide for this reason. Gortner (1949) states that "cellulose is the most widely distributed skeletal polysaccharide and the most abundant and chemically resistant of all substances elaborated by living cells."

Yonge (1926) stated that *Aplysia* digests cellulose, but he gave no evidence nor did he cite any particular species. The only other literature we found that refers to cellulases of *Aplysia* was Stone & Morton (1958). According to these authors, a weak cellulase was found in *Aplysia punctata* Cuvier, 1803 by Howells in 1942.

MATERIALS and METHODS

All *Aplysia vaccaria* used in this study were collected at Bird Rock, La Jolla, California (Lat. 32°49′ N; Long. 117°17′ W). They were packed in damp seaweed in plastic buckets with a small amount of sea water while being transported to the laboratory. Special care was taken not to expose the *Aplysia* to extreme sunlight while they were confined in a small volume of sea water.

Since *Aplysia vaccaria* is found very low in the intertidal zone, numbers of *Aplysia* sufficient for experimentation could be collected only when the tidal level was 0.5 feet or more below mean sea level. Few *Aplysia* could be found during turbulent surf conditions when the tide was changing, but they were often found grazing in the quietly receding waters an hour or so before low tide and about 30 minutes after the incoming tide had covered the area.

When the *Aplysia* could not be dissected within a few hours after being collected, they were kept in 20-gallon capacity aquaria filled with sea water. The water in these aquaria was continuously filtered and aerated.

Dissection of the *Aplysia* was necessary to remove fluid from the crop. Pipettes and syringes were tried unsuccessfully. Winkler's (1961) dissection method was used, then the circumesophageal nerve cords were cut and the ganglia extirpated.

Fluid was removed from the crop by pinching off the esophagus, severing it, then draining the crop contents into a beaker. The fluid portion was decanted into centrifuge tubes. Finer particles were removed by centrifugation at 29 000 g on a Model HT International centrifuge for 30 minutes.

Disintegration of Paper

A 6 ml sample of crop fluid obtained from 4 freshly collected *Aplysia vaccaria* which had been fed *Ulva* sp. was placed in a soft glass digestion tube along with a strip of Whatman No. 1 chromatography paper. The manufacturers of this paper state that it is 100% cellulose. No Pyrex glassware was used for holding incubation mixtures, since Pickford & Dorris (1934) have pointed out that some enzymes, i. e., trypsin and amylase, are inhibited by Pyrex glassware. Five drops of toluene

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were added as a bacteriostat.

A similar tube containing 6 ml of distilled water instead of crop fluid was also prepared. This tube also contained 5 drops of toluene and a strip of chromatography paper.

Both tubes were observed after 48 and 72 hours of incubation at room temperature (26° C), and the condition of the paper was noted.

Glucose Production from Paper

To determine whether or not cellulose is actually degraded to glucose by the crop fluid of *Aplysia vaccaria,* the following experiment was performed.

Fluid removed from the crops of freshly collected *Aplysia vaccaria* was centrifuged and placed into soft glass digestion tubes as follows:

Tube *T* contained 2 ml of crop fluid and a strip of chromatography paper. Tube *B* was prepared identically to tube *T* except that the crop fluid had been boiled for 10 minutes in a water bath. Tube *C* contained 2 ml of distilled water and a similar strip of chromatography paper. Tube *D* contained 2 ml of crop fluid, but no paper. Three drops of toluene were added to each tube as a bacteriostat, and each tube was stoppered with cotton. All tubes were incubated at room temperature (26° C) for 10 days.

The paper strips were removed from tubes *T, B,* and *C*. All tubes were centrifuged at 2 800 g for 10 minutes. The supernatants of the 4 tubes were tested for glucose using Glucostat reagent (Worthington Biochemical Corporation). The relative amounts of glucose present were indicated by the optical densities measured on a Model 15 Cary spectrophotometer at 400 mμ.

Determination of Optimal pH

In order to determine whether the glucose production observed was due to an enzymatic reaction or acid hydrolysis, an attempt was made to determine the optimal pH of the reaction.

Crop fluid of *Aplysia vaccaria* was obtained and centrifuged in a similar manner to that used in the preceding experiments.

Two sets of 10 non-Pyrex digestion tubes were prepared as follows. One ml of citrate-phosphate buffer prepared according to McIlvaine (1921) was added to each tube so that 2 identical series of 10 tubes were obtained. The 10 pH's tested were 2.2, 3.1, 4.1, 4.5, 5.1, 5.5, 6.0, 6.4, 7.0, and 7.9. These pH values were measured with a Beckman pH meter; all were within 0.2 of the pH expected from McIlvaine's formula. Identical strips of Whatman No. 1 chromatography paper were added to each tube. One ml of crop fluid was then added to each tube, and the paper was immediately removed from one series of 10 tubes. Four drops of toluene were added to each tube as a bacteriostat. The contents of all tubes were thoroughly mixed by rotating the tubes between the palms of the hands and then allowed to settle. After the toluene had separated and risen to the top, 0.3 ml of the fluid below the toluene was immediately assayed for glucose with Glucostat reagent. Glucose standards and a reagent blank were also assayed. All tubes were stoppered with cotton to prevent evaporation and locked in a dark cabinet. Care was taken to be sure no paper was exposed above the level of the toluene.

After 72 hours of incubation at room temperature (26° C), the contents of all tubes were again assayed for glucose in the same manner. Since the paper had started to disintegrate, the paper was removed, and the tubes were centrifuged at 2 800 g for 10 minutes before the assay was performed. After the assay, fresh paper strips were added. The tubes were restoppered with cotton and locked in a dark cabinet.

After another 72 hours of incubation, a total incubation time of 144 hours, the paper was removed, the tubes were recentrifuged, and the assay was repeated.

The entire experiment was repeated twice to obtain the values shown in Figures 2, 3, and 4.

RESULTS

Disintegration of Paper

After 48 hours of incubation, there was very little difference between the appearances of the paper strips, but by 72 hours of incubation, the paper in the tube containing crop fluid had disintegrated. The fibers had separated, and individual fibers were no longer distinct to the naked eye. Microscopic examination revealed some fibers similar in appearance to the original fibers, but about half as long. There was no observable change in the appearance of the paper strip in distilled water even after a week of incubation.

Similar results were obtained when the experiment was repeated 2 months later.

When *Ulva* sp. was substituted for paper in the above experiment, the results were inconclusive. *Ulva* in crop fluid disintegrated after 48 hours, but so did *Ulva* placed in a 50:50 mixture of sea water and McIlvaine's (1921) citrate-phosphate buffer at a pH of 5.5. *Ulva* sp. did not disintegrate in sea water or distilled water.

Glucose Production from Paper

The relative amounts of glucose present in each tube as indicated by the optical densities are shown in Figure 1. The optical density of the mixture prepared from tube *T* (crop fluid, paper, and toluene) and assayed with Glucostat was far higher than that of any of the control tubes.

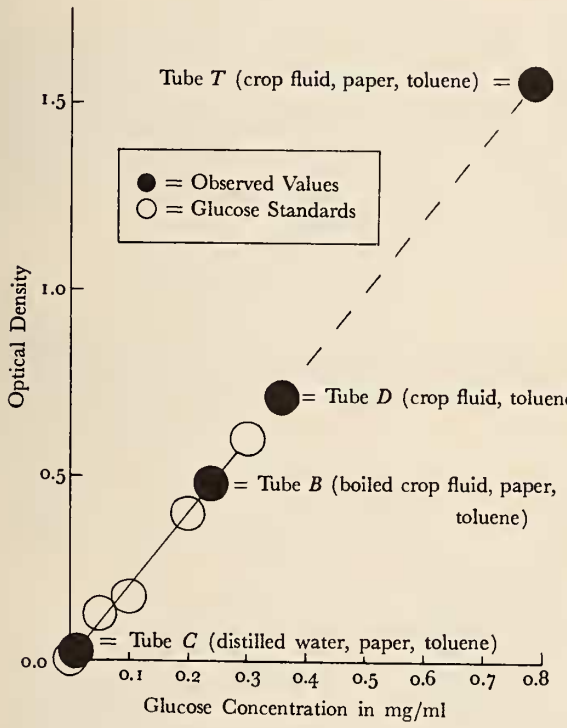

Figure 1

Glucose produced from filter paper substrate as indicated by optical density of glucostat

The low optical density of the mixture prepared from tube *B* (boiled crop fluid, paper, and toluene) suggests that glucose was not produced in that tube. A precipitate (probably denatured proteins) was observed during the boiling process. The absence of this precipitate from the mixture may account for the optical density being lower than that for the mixture prepared from tube *D*.

Glucose present in tube *C*, which contained distilled water instead of digestive fluid, appears negligible.

Since 2 of the observed optical densities were beyond the range of the standards for which Glucostat reagent is known to give a straight line relationship between glucose and optical density, no attempt was made to convert optical density to amount of glucose. Nevertheless, it is reasonable to state that the amount of glucose in tube *T* exceeded 0.30 mg per ml.

Determination of Optimal pH

Figures 2 through 4 graphically portray the results of this experiment. The optical density as measured on the spectrophotometer is an index of the amount of glucose present in each tube. The color of the crop fluid may also have contributed insignificantly to the optical density, but it would have been the same in all tubes, and only one-tenth of the assay mixture read by the spectrophotometer was crop fluid. Most of the points which did not fall near the curve of average values were affected by

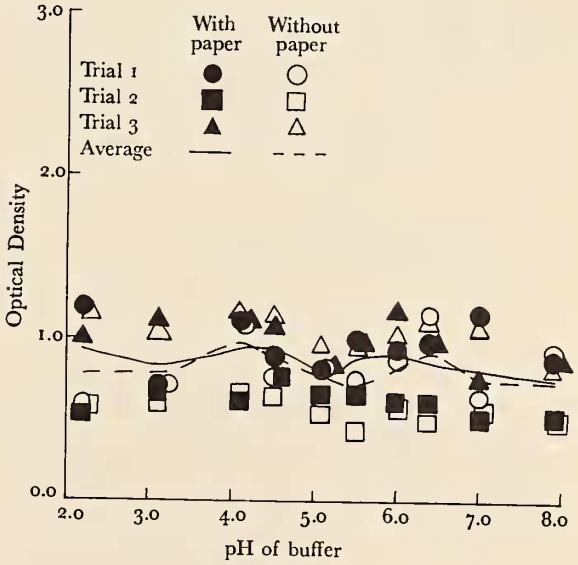

Figure 2

Optical density as an index of glucose present at T_0 (zero days of incubation)

turbidity differences in the incubation mixtures. However, the spectrophotometer could not differentiate between optical density and turbidity differences. As a result, the curves for the 3 individual trials are a bit more irregular than optical density differences alone would warrant.

The symbol T_0 indicates results obtained prior to any incubation period. Those results obtained after 3 days of incubation were labeled T_3. Results of assays made after 6 days of incubation were labeled T_6. Average values for the 3 trials are connected by a solid line curve

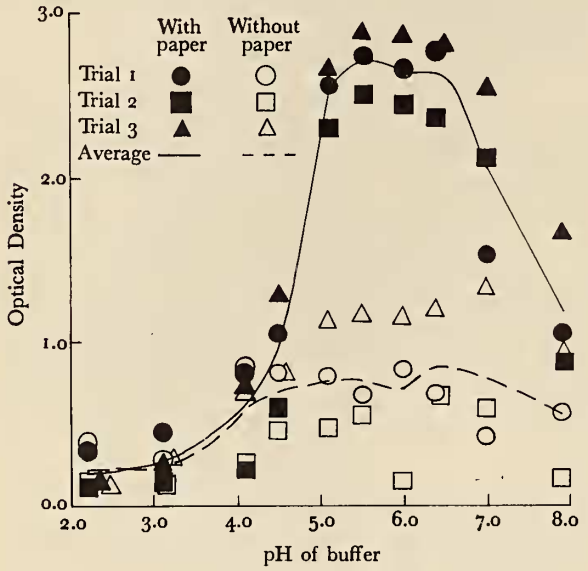

Figure 3

Optical density as an index of glucose present at T_3 (three days of incubation)

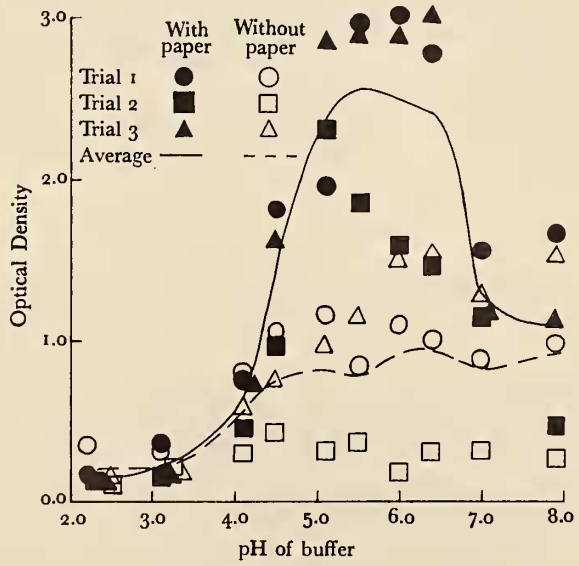

Figure 4

Optical density as an index of glucose present at T_6 (six days of incubation)

for the tubes with paper and by a dotted line curve for the tubes without paper.

It can readily be seen that there was no consistent difference between the curves at T_0. A pH optimum of about 5.5 was observed for the results taken at T_3 and T_6, 3 days and 6 days of incubation, respectively.

SUMMARY

The observation that chromatography paper disintegrates in crop fluid of *Aplysia vaccaria* does not prove that this animal is capable of utilizing the cellulose from its diet as a food source; however, it does suggest that more refined experiments should be performed to investigate the possibility that the opisthobranch may indeed possess this remarkable power.

Glucose Production from Paper

The results of this experiment do indicate that *Aplysia vaccaria* has the ability to convert paper (100% cellulose) to glucose, a source of energy utilized by most organisms. Since the boiled crop fluid in tube *B* did not yield a higher glucose reading than crop fluid alone (tube *D*), it suggests that the reaction is enzymatic. Apparently, boiling inactivates the enzyme or enzymes involved by denaturing them.

Determination of Optimal pH

The optimal pH of 5.5 observed for the production of glucose is good evidence that an enzyme or enzyme system is involved. If acid hydrolysis of the cellulose chains were the cause, one could expect the most glucose to be produced at the lowest pH's tested.

The pH optimum of 5.5 is a value consistent with other cellulases' pH optima. Parnas (1961) found one pH optimum of 5.75 for a cellulase from the crop of the snail *Levantina hierosolma* Boiss. He found another pH optimum of 5.6 for a cellulase from the hepatopancreas of the same snail. Lasker & Giese (1956) found cellulase pH optima of 4.0, 6.0, and 7.7 for the silverfish *Ctenolepisma lineata*. A pH optimum of 5.28 was determined for a cellulase of the terrestrial snail *Helix* by Karrer & Illing (1925).

Obviously a herbivorous mollusk that can convert cellulose to glucose has a distinct advantage over any competitors who lack this ability. *Aplysia vaccaria* seems to possess this ability. As yet, it is not proven that the cellulase is endogenous to *A. vaccaria,* i. e., the cellulase may be an extracellular enzyme produced by a bacterial

symbiont, but we do not believe this to be the case, on the basis of some preliminary bacterial studies.

Perhaps studies of other herbivorous mollusks will indicate that the phenomenon of cellulose digestion is not as unusual among the Mollusca as present knowledge indicates.

ACKNOWLEDGMENTS

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